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# ULTRASTRUCTURAL OBSERVATIONS ON HAEMOCYTOPOIESIS IN ADULT HORSESHOE CRAB (*TACHYPLEUS TRIDENTATUS*)*

HONG Shui-Gen
(*Laboratory of Cell Biology, Xiamen University, Xiamen* 361005, *China*)
HUANG Qin
(*The Swire Institute of Marine Science, The University of Hong Kong, Hong Kong*)
NI Zi-Mian
(*The Electron Microscope Unit, Xiamen University, Xiamen* 361005, *China*)

**Abstract** Haemocytopoiesis was studied in adult *Tachypleus tridentatus* by using transmission electron microscopy. Haemocytopoiesis occurred in connective tissues adjacent to the site of gametogenesis with basement membrane as a boundary between them. The haemocytopoiesis could be divided into three stages: prohaemocyte, plasmatocyte and granulocyte. In the male, haemocytes were developed in a reticular structure formed by boundary basement membrane which separated them from seminiferous tubules. The nucleus of early prohaemocyte was euchromatic, while heterochromatin arranged in nuclear periphery of late prohaemocyte, in which rich vesicles were distributed in the cytoplasm. The nuclei of plasmatocytes, which were larger than prohaemacytes, varied in shape as the cells did and contained rough, web-like heterochromatin. Cytoplasm of plasmatocytes looked empty and was low in electron density. There were a few protein-deposited vesicles in various sizes in the cytoplasm of late plasmatocytes. Granulocytes were rich in large granules about 1.5 μm in diameter and small granules about 0.5 μm in diameter. The number of granules increased along with the cell development. The mature haemocytes were 10~15 μm in size.

**Key words** Horseshoe crab (*Tachypleus tridentatus*), Haemocytopoiesis, Prohaemocyte, Plastmatocyte, Granulocyte, Basement membrane, Seminiferous tubules

The remarkable anti-bacterial function of horseshoe crabs (Arthropoda: Xiphosurida) haemocytes has attracted researchers' attention for over a century (Howell, 1885; Bang, 1956; Morimoto *et al.*, 1991; Ohta *et al.*, 1992; Suda *et al.*, 1995). The application of related researches is, however, facing a difficult situation because of the global decline in horseshoe crab populations (Galler, 1979; Rudloe and Rudloe, 1981; Holland, 1998). In

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**Brief introduction of the first author** HONG Shui-Gen, male, 58 years old, master of science, professor. Research subject: cell biology and developmental biology with especial reference to horseshoe crab. E-mail: shghong@xmu.edu.cn

the past decades, people have relied on horseshoe crabs for the manufacture of the biomedically important product Limulus Amoebocyte Lysate (LAL)(Armstrong, 1985; Mikkelsen, 1988; Rolbein, 1995). As the wild resource is declining, it is expected that LAL manufacturing can be continued with alternatives, including artificial breeding of the crabs (Kropach, 1979; Hong and Wu, 1985), collection of horseshoe crab haemocytes via laboratory cultivation (Chen *et al.*, 1989; Friberg *et al.*, 1992; Srimal, 1993) and clone of the genes which determine the LAL proteins and activities (Shigenaga *et al.*, 1990; Iwaki *et al.*, 1996; Holland, 1998).

The most critical problem in artificial breeding is the long life span of horseshoe crabs, e.g., *Tachypleus tridentatus* requires 13 to 14 years for its sexual maturation (Sekiguchi, 1988). The difficulty with the gene cloning program is also conspicuous in terms of the complex involved in the LAL reactions. In contrast, experiments for horseshoe crab haemocyte cultivation have been shown to be successful, except that the lysate from such cultivated cells did not demonstrate a sensitivity as high as that obtained in nature (Drummond, 1980; Chen *et al.*, 1989; Srimal, 1993).

In the haemocyte of a living horseshoe crab, the endotoxin-sensitive factors are known to be contained in various types of granules (Shishikura and Sekiguchi, 1979; Copeland and Levin, 1985; Suhr-Jessen *et al.*, 1989). Although ultrastructural studies suggest that these granules are developed in Golgi complex (Liang, 1985; Toh *et al.*, 1991), such knowledge needs to be improved by understanding haemocytopoiesis in horseshoe crabs (Armstrong, 1985). Liang *et al.* (1992) observed haemocytopoiesis in the embryos of *Tachypleus tridentatus* and described the morphology of early haemocytes. Cells with similar characters were identified from connective tissues in our previous studies of spermatogenesis in *T. tridentatus* (Hong *et al.*, 1995; Hong and Huang, 1999). In the present work, the morphology and distribution of such haemopoietic cells were re-examined to provide evidence of haemocytopoiesis in adult *T. tridentatus*.

## 1 Materials and Methods

Connective tissues of Tachypleus tridentatus were obtained from males with maximum carapace width varying between 17 and 22cm. Techniques for ultrastructural observations refer to Hong *et al.* (1995). Sections containing haemocytes at different developmental stages were examined and photographed using a JEM-100CX II transmission electron microscope. Although the nomenclature of invertebrate haemocytes underwent periodic modification in the past decades, the terms prohaemocyte, plasmatocyte and granulocyte have been used most commonly in the study of arthropod haemocytopoiesis since the 1970s (Ravindranath, 1981). These terms have, therefore, been applied to the present descriptions of haemocytopoiesis in the horseshoe crab *Tachypleus tridentatus*.

## 2 Results

According to the present transmission electron microscopic observations, haemocytopoiesis in adult *Tachypleus tridentatus* occurred in connective tissue's reticular structure formed by basement membrane that was in conjunction with the basement membrane of seminiferous tubules (Plate Ⅰ: 1, Plate Ⅱ: 2). An early prohaemocyte had a large nucleus which did not show heterochromatin (Plate Ⅰ: 1, Plate Ⅱ: 3). Heterochromatin appeared in late prohaemocyte along the periphery of the oval nucleus (Plate Ⅰ: 1, Plate Ⅱ: 2). Cytoplasm of prohaemocytes was rich in vesicles. Prohaemocytes were, on average, 10.5 $\mu$m along the longer axis and 3.5$\mu$m along the shorter axis (Plate Ⅱ: 3).

Plasmatocytes were larger than prohaemacytes, with the volume of cytoplasm increased (Plate Ⅰ: 1). Most cells were irregularly elongated. The nuclei of plasmatocytes varied in shape as the cells did and contained rough, web-like heterochromatin. The cytoplasm of plasmatocyte was shown to have a low electron density and had a few variously-sized vesicles which were deposited with proteins (Plate Ⅱ: 4). As shown in Plate Ⅰ: 1, late plasmatocytes, as well as granulocytes, were distributed among the matrix of connective tissues, indicating their involvement in the circulation. Neither prohaemocyte nor plasmatocyte was observed in division.

Early granulocytes contained many large granules which had an average diameter of 1.5$\mu$m and small granules, of about 0.6$\mu$m in diameter. The number of granules increased along with the cell development (Plate Ⅰ: 1, Plate Ⅱ: 4, 5). Golgi body and endoplasmic reticulum were involved in the formation of granules (Plate Ⅱ: 6). A mature amebocyte was often seen irregularly-shaped, 10 ~ 15$\mu$m in size and the heterochromatic nucleus was usually elliptic in longitudinal section, bearing nuclear pores of about 100nm in diameter (Plate Ⅱ: 6).

## 3 Discussion

Haemocytopoiesis in adult horseshoe crabs has remained one of the most interesting topics in the study of limulid amebocytes (Armstrong, 1985). Liang *et al*. (1992) for the first time described haemocytopoiesis in the embryos of *Tachypleus tridentatus*, showing that amebocytes were originated from exembryonic mesodermal cells and the formation of haemocytes did not occur until between 16 and 17 days after insemination, i.e., embryonic stage 13 according to Sekiguchi (1973).

In our present study, early haemocytes in adult *Tachypleus tridentatus* were shown to be developed in a connective tissue's reticular structure formed by basement membrane that was in conjunction with the basement membrane of seminiferous tubules. No dividing cells were, however, observed. Further investigations are needed to determine (1) the site for haemocytopoiesis in female *T. tridentatus*; (2) whether haemocytopoiesis in male *T. tridentatus* is restrict to seminiferous tubules or whether it occurs in other organs; (3) whether haemopoietic tissues in *T. tridentatus* are such organised as a particular organ; and (4) the origin and division status of early haemocytes in *T. tridentatus*. No specialised haemopoietic organs have been found in chelicerates (Sherman, 1981). Haemocytopoietic tissues are believed to be contained in the myocardial cell layers in some species of spiders (Seitz, 1972) and are presumed to be part of the lymphatic gland that attaches to cephalothoratic and preabdominal nerve cords in scorpions (Sherman, 1981).

Plasmatocytes are involved in the circulation of horseshoe crabs but in small quantities (Liang, 1985; Suhr-Jessen *et al*., 1989). Liang (1985) recognized a plasmatocyte as a premature form of granulocyte and found the cell to be capable of mitotic division in the blood. Suhr-Jessen *et al*. (1989) have, however, suggested that a plasmatocyte may be a granulocyte recovering from exocytosis although this, simultaneously, violated their conclusion that a plasmatocyte was not an exocytosed granulocyte. Results from our present observations are supportive of the description by Liang (1985) that a mature granulocyte was developed from a late plasmotocyte during circulation. This work also suggests that haemocytopoiesis in *Tachypleus tridentatus* is not restricted to the times of moulting, as assumed by Armstrong (1985) for *Limulus polyphemus*. The fact that prohaemocytes and early plasmatocytes are developed within the reticula of basement membranes may explain the absence of early haemocytes from circulatory system in horseshoe crabs (Armstrong, 1985; Suhr-Jessen *et al*., 1989).

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中　文　摘　要

# 中国鲎成体血细胞的发生

洪水根
（厦门大学细胞生物学研究室，福建厦门　361005）
黄　勤
（香港大学太古海洋科学研究所）
倪子绵
（厦门大学电镜室，福建厦门　361005）

应用透射电镜技术研究中国鲎成体血细胞发生。成体鲎血细胞发生于与生殖细胞发生相邻部位的结缔组织，其间以基膜为界。血细胞发生可分为原始血细胞、浆细胞和颗粒细胞3个阶段。在雄体，血细胞在由基膜形成的网状结构中发育，并以此为界与生精小管相邻。早期原始血细胞核以常染色质为主，异染色质仅在晚原始血细胞核内边缘出现，胞质充满大小不一的膜囊。浆细胞比原始血细胞大，细胞及核的形状多样，核异染色质呈粗网状分布，胞质空虚、电子密度低。晚浆细胞胞质逐渐出现少量膜囊，蛋白质开始在其中沉积。颗粒细胞胞质富含直径约1.5μm的大颗粒和直径0.5μm左右的小颗粒，其数量随细胞发育而增加。成熟鲎血细胞大小为10～15μm。

关键词　中国鲎　血细胞发生　原始血细胞　浆细胞　颗粒细胞　基膜　生精小管

# Explanation of Plates（图 版 说 明）

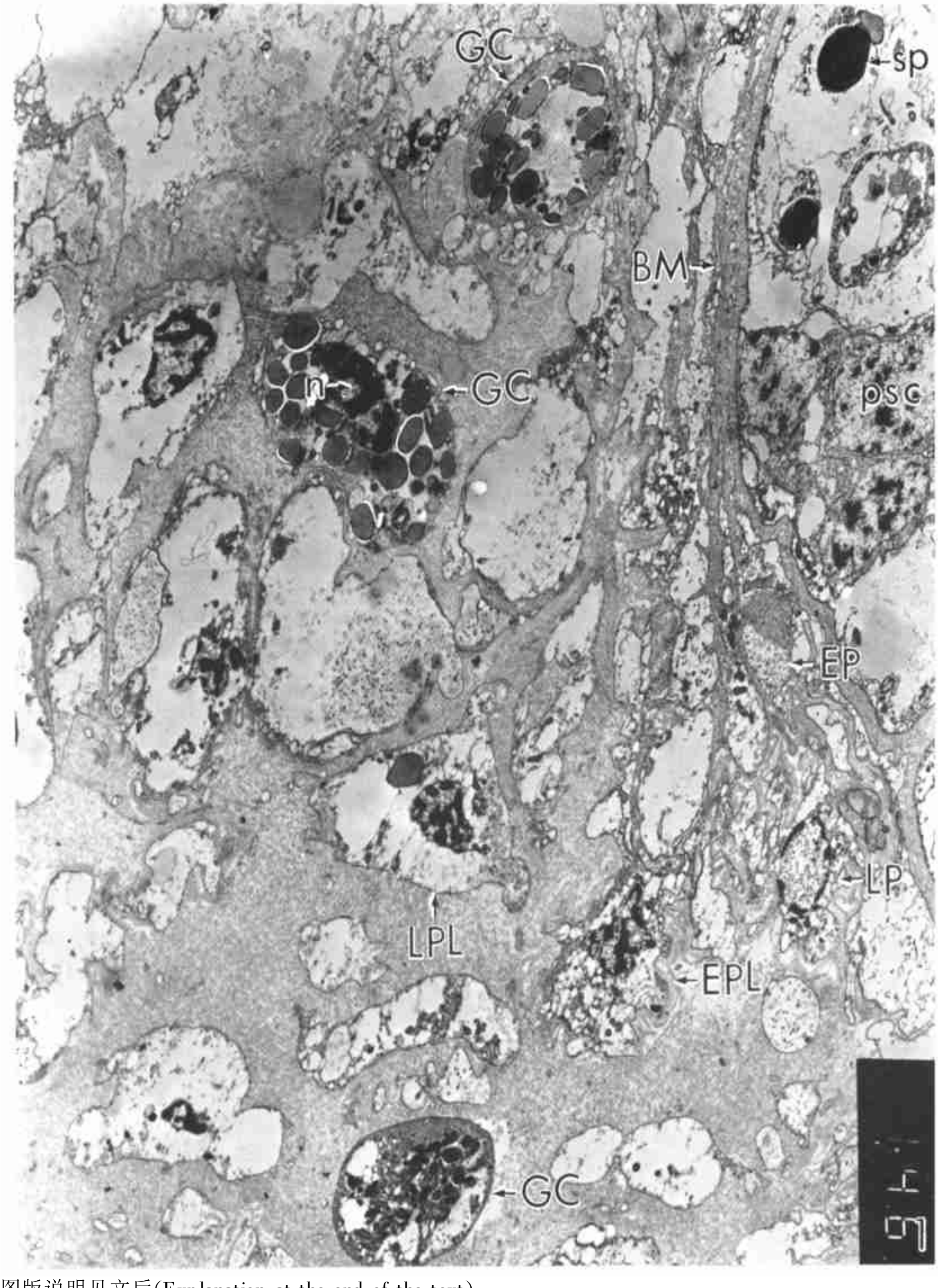
## Plate Ⅰ（图版 Ⅰ）

1. Low magnification survey of connective tissues that attach to seminiferous tubules and haemocytopoietic reticula in *Tachypleus tridentatus*. Prohaemocyte and young plasmatocyte were distributed in the haemocytopoietic reticula. Late plasmatocytes and granulocytes were distributed among matrix, Arrow indicates conjunctions between basement membranes of haemocytopoietic tissues and seminiferous tubules.（中国鲎与生精小管相邻的造血网结缔组织电镜低倍观。造血网中可见原始血细胞和幼浆细胞。晚浆细胞及颗粒细胞分布于基质中。箭头所指为造血组织与生精小管两者之间基膜相连的部分）×6 500

## Plate Ⅱ（图版 Ⅱ）

2. Electron micrograph of haemopoietic reticula from *Tachypleus tridentatus*. The reticular structure is formed by basement membrane and the arrow indicates a nucleus of late prohaemocyte.（鲎造血网电镜照片，示由基膜形成的网状结构。箭头示晚原始血细胞的核）×5 800
3. An early prohaemocyte of *Tachypleus tridentatus* existed between two basement membranes. There was no heterochromatin in the nucleus and the cytoplasm was rich in vesicles.（一个位于两层基膜之间的鲎早原始血细胞。核为常染色质，胞质充满大量大小不一的膜囊）× 7 200
4. A late plasmatocyte and an early granulocyte from *Tachypleus tridentatus*. In the plasmatocyte, the nucleus had rough, web-like heterochromatin. Arrow indicates protein deposit in a vesicle. There were large and small granules in the granulocyte.（一个晚浆细胞和一个早颗粒细胞。浆细胞中，核具粗网状的异染色质，箭号指蛋白质在膜囊中沉积。而在颗粒细胞中，大颗粒和小颗粒开始出现）×5 800
5. A granulocyte which contains many large granules and small granules.（颗粒细胞，胞质中充满许多大颗粒和小颗粒）×4 800
6. Magnification view of a mature hemocyte, showing nuclear pore and the relationship between the formation of granules and Golgi body, endoplasmic reticulum.（一个成熟血细胞的放大观。示胞质中的核孔及颗粒形成与高尔基体及内质网的关系）×14 000

BM：basement membrane（基膜） EP：early prohaemocyte（早原始血细胞） EPL：early plasmatocyte（早浆细胞） G：Golgi body（高尔基体） GC：granulocyte（颗粒细胞） L：large granule（大颗粒） LP：late prohaemocyte（晚原始血细胞） LPL：late plasmatocyte（晚浆细胞） M：mitochondria（线粒体） N：nucleus（细胞核） NP：nuclear pore（核孔） PS：primary spermatocyte（初级精母细胞） RER：rough endoplasmic reticulum（粗面内质网） S：small granule（小颗粒） SP：spermotozoon（精子） V：vesicle（膜囊）

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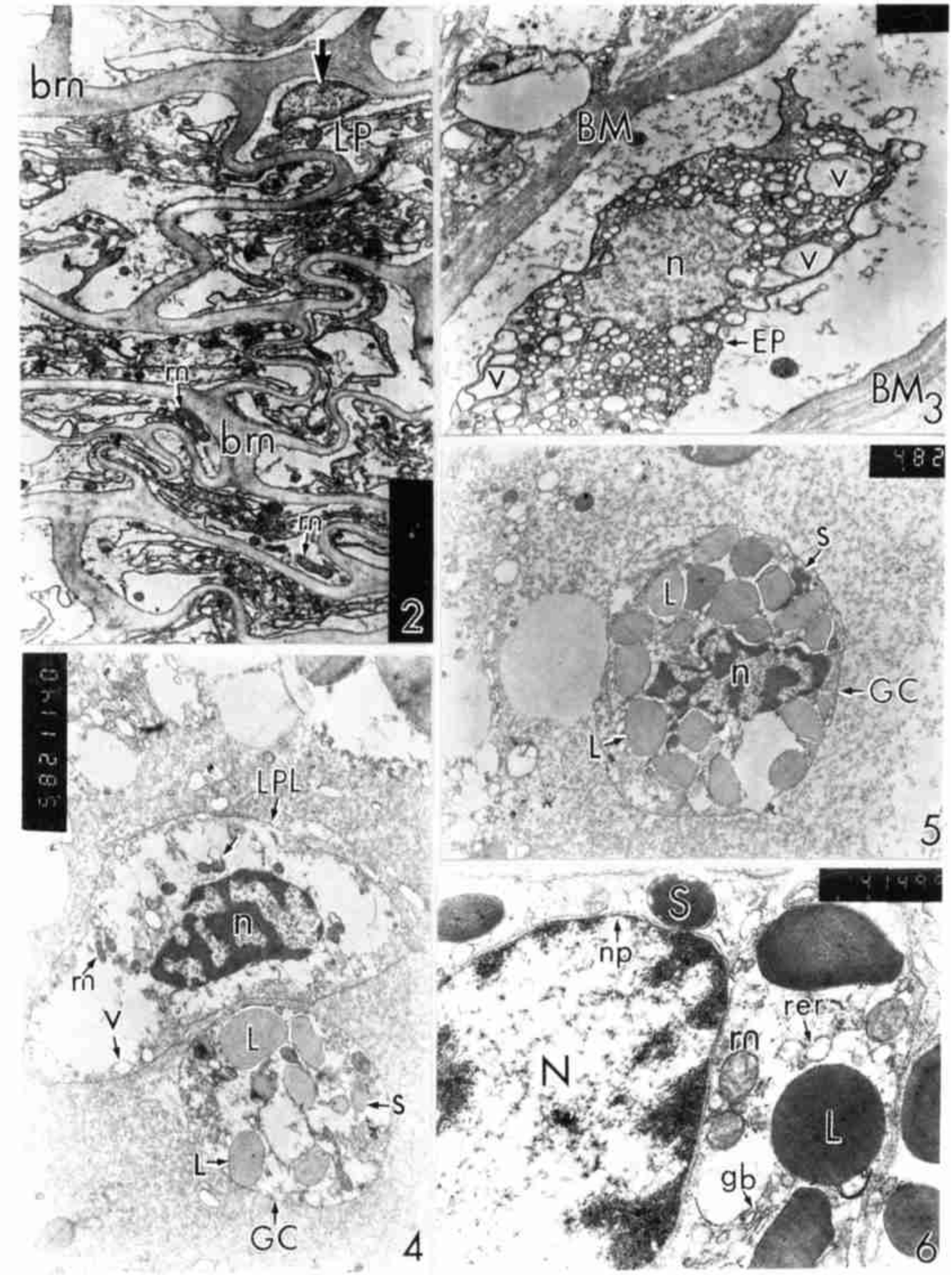


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